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(21) International Application Number: PCT/DK99/00245 (22) International Filing Date: 4 May 1999 (04.05.99) (30) Priority Data: 0615/98 4 May 1998 (04.05.98) DK (71) Applicant (for all designated States except US): DAKO A/S [DK/DK]; Produktionsvej 42, DK-2600 Glostrup (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): PLUZEK, Karl-Johan [DK/DK]; Primulahaven 38, DK-2765 Smørum (DK). NIELSEN, Kirsten, Vang [DK/DK]; Rugvej 7, DK-2700 Brønshøj (DK). ADELHORST, Kim [DK/DK]; Solbakken 38, DK-2840 Holte (DK). (74) Agent: HOFMAN-BANG & BOUTARD, LEHMANN & REE A/S; Hans Bekkevolds Allé 7, DK-2900 Hellerup (DK).		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: METHOD AND PROBES FOR THE DETECTION OF CHROMOSOME ABERRATIONS		
(57) Abstract A novel method for detecting chromosome aberrations is disclosed. More specifically, chromosome aberrations are detected by <i>in situ</i> hybridisation using at least two sets of hybridisation probes, at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to a potential aberration in a chromosome, and at least one set comprising two or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to another potential aberration in a chromosome. In particular, the method may be used for detecting chromosome aberrations in the form of breakpoints.		

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METHOD AND PROBES FOR THE DETECTION OF CHROMOSOME ABERRATIONS

The present invention relates to a novel method for
5 detection of chromosome aberrations. More specifically,
the present invention relates to the determination of
chromosome aberrations by *in situ* hybridisation using at
least two sets of hybridisation probes, at least one set
comprising one or more peptide nucleic acid probes
10 capable of hybridising to specific nucleic acid sequences
related to a potential aberration in a chromosome, and at
least another set comprising one or more peptide nucleic
acid probes capable of hybridising to specific nucleic
acid sequences related to another or the same potential
15 aberration in a chromosome. In particular, the present
invention relates to the determination of chromosome
aberrations by *in situ* hybridisation using at least two
sets of hybridisation probes, at least one set comprising
one or more peptide nucleic acid probes capable of
20 hybridising to specific nucleic acid sequences flanking
one side of a potential breakpoint in a chromosome, and
at least another set comprising two or more peptide
nucleic acid probes capable of hybridising to specific
nucleic acid sequences flanking the other side of the
25 potential breakpoint in the chromosome. The invention
further relates to such sets of hybridisation probes.

BACKGROUND OF THE INVENTION

30 Chromosome aberrations are well recognised to play an
important role as a cause for diseases, not least as a
cause for malignant diseases. Detection of such
aberration is, therefore, an important target for novel
diagnostic tools. Well known techniques known in the arts
35 of molecular biology and chromosome analyses are thus
currently applied for detection of these important causes

of diseases. So far, however, a fast, convenient, cheap and user-friendly test which allows for widespread and routine detection of chromosome aberrations has not been available.

5

According to i.a. the international patent application, publication no. WO 93/24652 (ref. 1) it has been found that peptide nucleic acids hybridise fast, selectively and strongly to nucleic acids such as, for example,
10 chromosomes or nucleic acids derived from chromosomes.

According to the international patent application, publication no. WO 97/18325 (ref. 2) i.a. chromosome aberrations can be detected by hybridising peptide
15 nucleic acids to chromosomes or fragments thereof followed by detection of the binding pattern of the peptide nucleic acids.

An area of particular interest regarding chromosome
20 aberration are the so called breakpoints that have been demonstrated in numerous cases to be specifically linked to the development of malignant diseases.

SUMMARY OF THE INVENTION

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In the broadest aspect, the present invention provides a method of determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in situ* hybridisation, comprising the steps of
30

- a) producing a preparation of the sample, whereby the sample will be subject to a fixation,
- b) contacting said preparation with a hybridisation
35 solution comprising at least two sets of hybridisation probes, at least one set comprising one

or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to a potential aberration in a chromosome, and at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to another or the same potential aberration in a chromosome, and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and

d) determining the presence of peptide nucleic acid probe in the preparation.

20

In particular, the present invention enables the determination of chromosome aberrations in the form of breakpoints.

25 Thus, the present invention further provides a method of determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in situ* hybridisation, comprising the steps of

30 a) producing a preparation of the sample, whereby the sample will be subject to a fixation,

b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes one set comprising, at least one or more peptide nucleic acid probes capable of

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hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and

d) determining the presence of peptide nucleic acid probe in the preparation.

In a further aspect, the present invention provides a method of determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in situ* hybridisation, comprising the steps of

a) producing a preparation of the sample, whereby the sample will be subject to a fixation,

b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes one set comprising, one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other

side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

- c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and
- d) determining the presence of peptide nucleic acid probe in the preparation.

In WO 97/18325 (ref. 2), a method for determining the presence of specific nucleic acid sequences in a sample of eukaryotic origin using *in situ* hybridisation is disclosed. The method comprises the steps of producing a preparation of the sample to be investigated, contacting the preparation with a hybridisation solution comprising at least one peptide nucleic acid probe and a hybrid destabilising agent, removing any unbound and non-specifically bound probe, and determining the presence of bound probe. The method may i.a. be used for detection of X chromosome specific sequences or centromeric sequences. In comparison, the present invention is based on a different concept, namely the use of at least two sets of hybridisation probes capable of hybridising to sequences of at least two different regions of chromosomes related to the same potential chromosome aberration or in different potential chromosome aberrations, thereby enabling determination of chromosome aberrations.

In DE 196 10 255 (ref. 3), nucleic acid sequences are disclosed, which sequences are specific for a translocation breakpoint of a chromosome. The sequences comprise a first set of sequences complementary to a DNA

sequence of a first chromosome, which first set is capable of hybridising to the first DNA sequence, and a second set of sequences complementary to a DNA sequence of a second chromosome, which second set is capable of
5 hybridising to the second DNA sequence. Only nucleic acid probes are mentioned. The use of peptide nucleic acid probes is not mentioned, nor anticipated.

EP 727 487 (ref. 4) and EP 825 198 (ref. 5) relate to
10 Multi-tumour Aberrant Growth (MAG) genes and genes having a sequence of any one of the strands of any one of the members of the PLAG subfamily of zinc finger protein genes or CTNNB1 genes, respectively. Various methods for detecting chromosome aberrations are also described. The
15 concept of the methods described in these two documents is different from the concept of the present invention. The methods disclosed in EP 727 487 (ref. 4) and EP 825 198 (ref. 5), respectively, are based on a comparison between the aberrant gene and the wild-type gene, whereas
20 the method of the present invention enables direct detection of the potential chromosome aberration or aberrations. Furthermore, the use of peptide nucleic acid probes is not disclosed, nor anticipated.

25 DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the present invention relates to a method for determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in*
30 *situ* hybridisation, in which method at least two sets of hybridisation probes of peptide nucleic acid probes capable of hybridising to sequences related to the potential aberration or aberrations are used.

The method is suitable for detecting sequences related to a broad range of potential aberrations, and thereby determining whether aberrations are present.

- 5 The sets of hybridisation probes may be used for determining nucleic acid sequences related to different chromosome aberrations or the same aberration.

Thus, in a particular embodiment of the present method,
10 the potential aberration is a potential breakpoint.

Other examples of such potential aberrations are deletions, amplifications, inversions, duplications and aneuploidy.

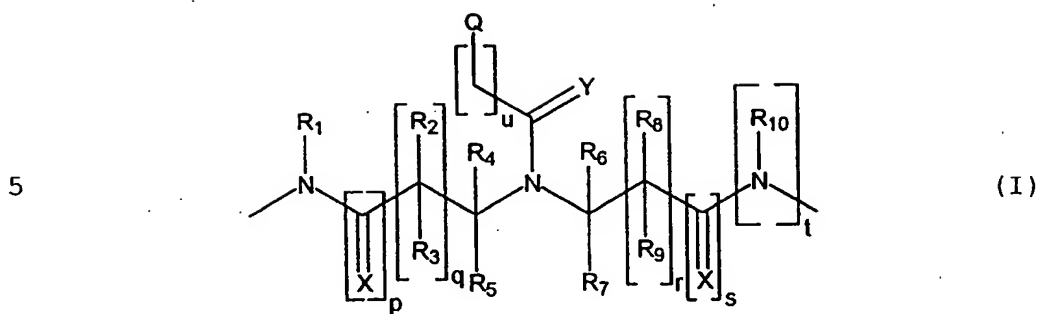
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Furthermore, the present invention relates to a method for determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in situ* hybridisation, in which method at least two sets of
20 hybridisation probes of peptide nucleic acid probes flanking a potential breakpoint in a chromosome are used.

Suitable peptide nucleic acid probes to be used in the present invention are such containing from 8 to 40 polymerised moieties such as from 8 to 30 polymerised
25 moieties.

Preferably, in the case of breakpoints, the sets of hybridisation probes should be chosen so as to hybridise
30 to said specific nucleic acid sequences flanking a potential breakpoint in a chromosome within a genomic distance of no more than 100 kb, preferably no more than 50 kb from the potential breakpoint.

35 The probes are suitably such comprising polymerised moieties of formula (I)



wherein

10

Y designates O or S,

each X independently designates O or S,

each Q independently designates a ligand that is a naturally occurring nucleobase, a non-naturally occurring nucleobase, an intercalator, a nucleobase-binding group, a label or H,

15

u is an integer from 0, or 1 to 5,

p and s independently designate 0 or 1,

q and r independently designate 0 or 1,

20

t designates 0 or 1,

R₁ and R₁₀ independently designate H or C₁₋₄ alkyl,

R₂, R₃, R₄, R₅, R₆, R₇, R₈ and R₉ independently designate H, the side chain of a naturally occurring amino acid, or the side chain of a non-naturally occurring amino acid.

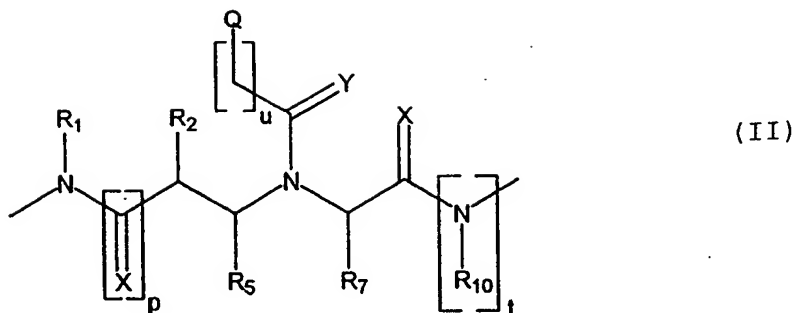
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Suitable probes are such wherein Y, X, Q, u, p, q, r, s, R₂, R₅, R₇ and R₈ are as defined above, t is 0, R₁ designates H or CH₃, and each of R₃, R₄, R₆ and R₉ designates H.

30

Peptide nucleic acid probes comprising polymerised moieties of formula (II), which are moieties of the general formula (I) wherein r is 0 and q and s are 1,

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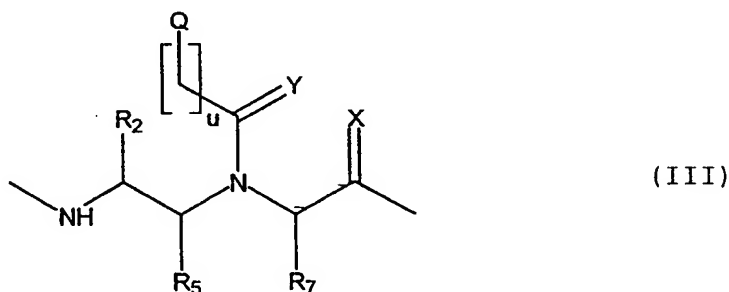


wherein Y, X, Q, p, t, u, R₂, R₅ and R₇ are as defined in claim 4, and

R₁ and R₁₀ independently designate H or CH₃,

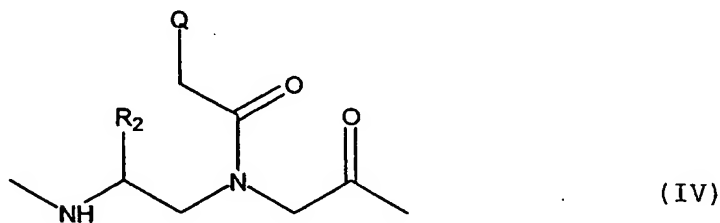
10 are suitable for use in the present invention.

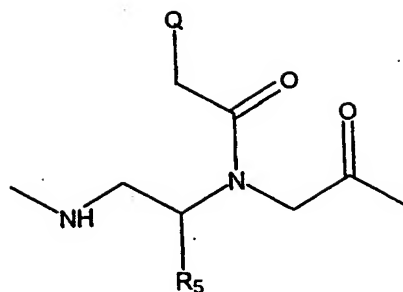
In particular, probes comprising polymerised moieties of formula (III), which are moieties of the general formula (I) wherein p, r and t are 0, and q and s are 1



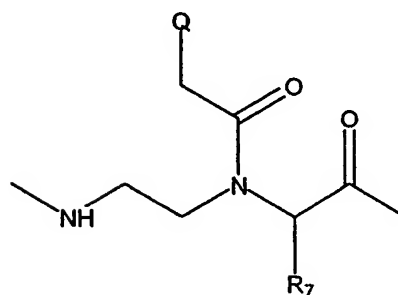
wherein Y, X, Q, u, R₂, R₅ and R₇ are as defined above, may be used.

25 In a special embodiment, peptide nucleic acid probe comprising polymerised moieties of formulas (IV), (V) and/or (VI), which are moieties of the general formula (I) wherein p, r and t are 0, and u, s and q are 1,





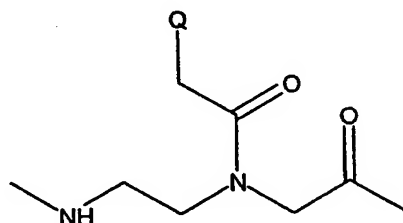
(V)



(VI)

wherein R₂, R₅ and R₇ are as defined above, and each Q
 15 independently designates a naturally occurring
 nucleobase, a non-naturally occurring nucleobase, an
 intercalator or a nucleobase-binding group, may be used.
 In particular, R₂, R₅ and R₇ may designate H or the side
 chain of Ala, Asp, Cys, Glu, His, HomoCys, Lys, Orn, Ser
 20 or Thr, and Q designates thymine, adenine, cytosine,
 guanine, uracil or hypoxanthine.

Preferred peptide nucleic acid probe are those comprising
 polymerised moieties of formula (VII), which are moieties
 25 of formula (I) wherein p, r and t are 0, and u, s and q
 are 1



(VII)

35 wherein Q designates thymine, adenine, cytosine, guanine,
 uracil or hypoxanthine.

The method of the invention can be used for detecting a broad range of chromosome aberrations, and further can be used for the diagnosis of disorders and diseases related to chromosomal aberrations or abnormalities such as e.g. haemopoietic malignancies, cancers and inborn constitutional diseases. Furthermore, the method may be used for detecting viral sequences and their localisation in the chromosome.

10

The term "chromosome aberration" is intended to mean translocation, amplification, inversion, duplication and aneuploidy.

15 The term "nucleic acid sequences related to a potential aberration in a chromosome" is intended to comprise a region and regions of a chromosome/chromosomes of interest in relation to the aberration or aberrations in question.

20

Examples of chromosome aberration-related disorders or diseases are acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemias, non-Hodgkin's lymphomas (NHL), malignant lymphomas, and multiple lymphomas. Well-established chromosome aberrations have been identified in many events. Often the particular chromosome aberration can be used to forecast the prognosis as some genetic aberrations are associated with favourable prognosis, whereas others are associated with poor prognosis.

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Examples of other chromosome aberrations related to solid tumours and cancers are HER-2 (ERBB2) gene amplification frequently related to breast cancers. A breast cancer panel may also include e.g. CMYC (c-myc) amplification, EGFR (epidermal growth factor receptor) amplification or

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duplication, amplification of genes localised to chromosome 20q13, TP53 (p53) deletion, and RB deletion. For other cancer types similar panels may be chosen. Also, some of the aberrations mentioned occur in other
5 cancer types.

Lymphoid malignancies are manifested in a broad range of diseases which differ in clinical symptoms, prognosis and treatment regimen. Such malignancies can be divided into
10 B-cell malignancies and T-cell malignancies. ALL can further be classified as pro-B-ALL, common-ALL, pre-B-ALL and additional T-ALL types.

In the case of ALL and NHL, several different types of
15 chromosome aberrations have been identified. For example, translocations resulting in fusion genes encoding for fusion proteins with new or modified functions have been identified in precursor-B-ALL (e.g. E2A-PBX and BCR-ABL fusion proteins from t(1;19) and t(9;22), respectively).
20 In acute leukemias, the 11q23 region with the MLL gene is important. Other examples are t(14;18) with involvement of the BCL2 gene, t(11;14) with involvement of the BCL1/Cyclin D1 gene, TAL1 deletions in T-ALL, t(12;21) in precursor-B-ALL, and t(2;5) in anaplastic large cell
25 lymphoma. Further examples are t(4;11)(q21;q23) involving the MLL-AF4 gene, t(11;19)(q23;p13) involving the MLL-ENL gene, t(6;11)(q27;q34) involving the MLL-AF6 gene, and t(9;11)(p22;q23) involving the MLL-AF9 gene in the case of acute leukemias, t(2;18)(p12;q21) involving the IGK-BCL2 gene, and t(18;22)(q21;q11) involving the IGL-BCL2
30 gene in the case of malignant lymphomas, and t(11;22)(q24;q12) involving the EWS-FLI1 gene, t(21;22)(q22;q12) involving the EWS-ERG gene, and t(7;22)(p22;q12) involving the EWS-ETV1 gene in the case
35 of solid tumours.

In the method of the invention each peptide nucleic acid probe is suitably labelled directly or indirectly with at least one detectable label such as those selected from the group consisting of enzymes, chromophores, fluorochromes, and haptens. Furthermore, various systems for enhancing or amplifying the signal may also be applied. Such systems are well-known in the art.

In a preferred embodiment of the invention labels are attached to the peptide nucleic acids in such a fashion that all peptide nucleic acids of one set of probes is labelled with one specific label while the peptide nucleic acids of the other set are labelled with another specific label.

For example, in the case of detection of breakpoints the use of different labels is an interesting option. By monitoring the fluorescence after hybridising the two sets of probes to the nucleic acid target to be investigated the presence or absence of a breakpoint is readily apparent from the combined fluorescence of the peptide nucleic acid probe sets: if the two fluorescent signals are fused no break has occurred while the presence of two separate signals from each set of peptide nucleic acid probes indicates, that a break has occurred between the target regions or targets investigated.

The method of the invention can be carried out using various forms of detectable labels and detection methods. Also, the method of the invention may be utilised for simultaneous detection of several aberrations by employing several sets of peptide nucleic acid probes.

Peptide nucleic acids to be used according to the method of the invention can be selected on the basis of sequence analysis of nucleic acid regions to be investigated. The

peptide nucleic acids can be synthesised and labelled according to methods known in the art.

5 Hybridisation of the peptide nucleic acid sets to target material under investigation can be carried out for example as exemplified in the international patent application no. WO 97/18325 (ref. 2). Subsequent detection of the peptide nucleic acid hybridisation pattern is carried out according to methods well known in
10 the art.

The hybrid destabilising agent is present in an amount effective to decrease the melting temperature of hybrids formed between the nucleic acid to be determined and the
15 peptide nucleic acid probe used to detect said nucleic acid so as to increase the ratio between specific binding and non-specific binding. Within the present context, specific binding is intended to mean that the occurrence of false positive signals due to binding of the probes to
20 other nucleic acid sequences having the same or similar sequence as the target sequence is reduced sufficiently to ensure determination under the used conditions.

Examples of hybrid destabilising agents are formamide,
25 urea, ethylene glycol, guanidine and glycerol. Most of these agents may preferably be present in an amount of above 10% and less than 80%, such as 70%. Formamide and guanidine may more preferably be present in an amount of from 20% to 80%, more preferably of from 20% to 60%, most
30 preferably of from 30% to 70% or from 30% to 50%. Urea is preferably used in a concentration from 2 to 5 M. Ethylene glycol may more preferably be present in an amount of from 30% to 65%, most preferably of from 50% to 65%.

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The present invention provides a method for determining the presence of specific nucleic acid sequences in samples of eukaryotic origin, such as of human, animal or plant origin. Non-limiting examples are tissue sections,
5 cell smears, suspensions of cells or parts thereof and chromosome spreads. The presence of viral and/or bacterial DNA as well as non-eukaryotic DNA incorporated into eukaryotic DNA also may be detected using the present method.

10

The peptide nucleic acid probe may suitably be labelled or unlabelled. In one aspect, the peptide nucleic acid probe carries one or more labels for detection of hybrids formed during hybridisation.

15

In one embodiment of the present invention, a washing buffer at alkaline pH may preferably be used in step (c). The use of a washing buffer having an alkaline pH may in some cases provide an increased ratio between specific
20 binding and non-specific binding.

In another aspect, the present invention concerns a pair of set of hybridisation probes, one set hybridising to specific nucleic acid sequences related to a potential
25 aberration of a chromosome, and one set hybridising to specific nucleic acid sequences related to another or the same potential aberrations of a chromosome, each set comprising one or more peptide nucleic acid probes of formula (I) to (VII). In one embodiment, the pair of sets
30 is such, wherein the sets are capable of hybridising to nucleic acid sequences related to different chromosome aberrations. In another embodiment, the pair of sets is such which comprises sets of hybridisation probes capable of hybridising to different nucleic acid sequences
35 related to the same potential aberration. An example of

the latter is sets of probes capable of hybridising to nucleic acid sequences related to a potential breakpoint.

The invention further relates to a pair of sets of hybridisation probes, one set hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome and another set hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and each set comprising one or more peptide nucleic acid probes of formula (I) to (VII). The pair of sets of hybridisation probes is preferably such wherein each set hybridises to specific nucleic acid sequences flanking either side of a potential breakpoint in a chromosome within a genomic distance of no more than 100 kb, preferably no more than 50 kb from the potential breakpoint.

The pair of sets of hybridisation probes according to the invention is preferably such in which each set comprises from 1 to 500, from 1 to 250, from 1 to 100, from 1 to 35 peptide nucleic acid probes.

The invention also relates to an *in situ* diagnostic method for diagnosing chromosome aberrations in a sample of eukaryotic origin comprising the steps of

- a) producing a preparation of the sample, whereby the sample will be subject to a fixation,
- b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to a potential aberration in a chromosome, and at least another set comprising one or more

peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to another or the same potential aberration in a chromosome, and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and

d) determining the presence of peptide nucleic acid probe in the preparation.

In particular, one embodiment of the diagnostic method is an *in situ* diagnostic method for diagnosing chromosome aberrations in a sample of eukaryotic origin comprising the steps of

a) producing a preparation of the sample, whereby the sample will be subject to a fixation,

b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature

of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

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c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and

d) determining the presence of peptide nucleic acid probe in the preparation.

10

In a further embodiment of the diagnostic method, the method concerns an *in situ* diagnostic method for diagnosing chromosome aberrations in a sample of eukaryotic origin comprising the steps of

15

a) producing a preparation of the sample, whereby the sample will be subject to a fixation,

b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

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- c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and
- d) determining the presence of peptide nucleic acid probe in the preparation.

The invention further concerns the use of a pair of sets of hybridisation probes as defined above for use in the method of the invention, and for use in the *in vitro* diagnostic method of the invention.

Starting material

The present method can be used to detect nucleic acid sequences in samples of eucaryotic origin. It is contemplated that the present method provides a valuable tool for analysing such samples for the presence of nucleic acid sequences specific for e.g. pathogenic bacteria or virus hence providing information for establishing a diagnosis. In human or animal pathology, the detection of chromosomal aberrations may provide clinically important information for diagnosing genetic disorders or diseases. In plant biology, it is further contemplated that the present method may be a valuable tool for monitoring the efficiency of transferring for example herbicide resistance genes to a plant or, like in human tissues, to establish specific synthesis of proteins (by detection of mRNA) in a given cell structure.

The term "sample of eukaryotic origin" includes, but is not limited to samples of human, animal or plant origin. In the present context, the term "sample" is intended to include, but is not limited to, human, animal or plant tissue sections, cell or tissue cultures, suspension of human, animal or plant cells or isolated parts thereof,

human or animal biopsies, blood samples, saliva, urine, cerebrospinal fluid, milk, excretions, secretions, swabs, faecal samples and aspirates.

5 Producing a preparation of the sample

The sample to be examined is pre-treated before the hybridisation step whereby a preparation of the sample is produced. The person skilled in the art will readily
10 recognise that the appropriate pre-treatment will depend on the type of sample to be examined. During the pre-treatment, the sample will be subject to a fixation.

Thus, in one embodiment of the method, the sample is
15 deposited onto a solid support. Techniques for depositing a sample onto the solid support will depend upon the type of sample in question and may include, for example, sectioning of tissue as well as smearing or cytocentrifugation of cell suspensions. Many types of
20 solid supports may be utilised to practice the present method. The use of such supports and the procedures for depositing samples thereon are known to those skilled in the art. Glass microscope slides are especially convenient. Glass microscope slides can be treated to
25 better retain the sample.

Prior to hybridisation, the sample is suitably pre-treated with various chemicals and/or enzymes to facilitate the subsequent reactions. The actual pre-
30 treatment will depend on the type of sample to be analysed. The preferred treatment is one which fixes and preserves the morphological integrity of the cellular matrix and of the nucleic acids within the cell as well as enables the most efficient degree of probe
35 penetration.

In producing a preparation of a tissue sample, the morphological integrity of a tissue and the integrity of the nucleic acids can be preserved by bringing the sample to a fixed stage either by means of chemical fixation or freezing. When freezing is used for preservation of for instance a biopsy, the biopsy is typically frozen in liquid nitrogen. After freezing, the sample may appropriately be stored at -80 °C. Prior to the analysis of the nucleic acid, the frozen sample is cut into thin sections and transferred to e.g. pre-treated slides. This can e.g. be carried out at a temperature of -20 °C in a cryostat. The biopsy or tissue sections may suitably be stored at -80 °C until use. Prior to hybridisation, the tissue section may be treated with a fixative, e.g. a precipitating fixative such as acetone and/or the tissue section is incubated for a short period in a solution of buffered formaldehyde. Alternatively, the biopsy or tissue section can be transferred to a fixative such as buffered formaldehyde for 12 to 24 hours. Following fixation, the tissue may be embedded in paraffin forming a block from which thin sections can be cut. Well prepared paraffin-embedded samples can be stored at room temperature for a period of years.

Prior to hybridisation, the tissue section is deparaffinated and re-hydrated using standard procedures.

Further permeabilisation may be necessary in order to ensure sufficient accessibility of the target nucleic acid sequences to the peptide nucleic acid probe. The type of treatment will depend on several factors, for instance on the fixative used, the extent of fixation, the type and size of sample used and the length of the peptide nucleic acid probe. The treatment may involve exposure to protease such as proteinase K, pronase or

pepsin, diluted acids, detergents or alcohols or a heat treatment.

5 In some cases, a pre-hybridisation step using a pre-hybridisation mixture very similar to the hybridisation solution but without the peptide nucleic acid probe or another pre-hybridisation solution without probe, might be useful in order to decrease non-specific binding of the peptide nucleic acid probe. The components of the
10 pre-hybridisation mixture should be selected so as to obtain an effective saturation of sites in the tissue that might otherwise bind the peptide nucleic acid probe non-specifically.

15 For analysing a suspended preparation such as a suspension of cells, the sample is treated so as to obtain a permeabilisation of the material and a preservation of the morphology. Fixation may be carried out with a fixative such as formaldehyde, acetone or
20 ethanol.

The present method permits the detection of a specific nucleic acid sequence in a chromosome. Such detection may for example be carried out using spreads of chromosomes
25 in metaphase, where the target sequence may be located in any region of the chromosomes. A sample preparation may in this case comprise adding an agent such as colcemide to arrest the chromosomes of dividing cells in the metaphase of the mitosis. Subsequently, the sample may be
30 treated with a hypotonic buffer. Following centrifugation, the chromosomes are treated with a fixative and spread on a slide. Immediately before or simultaneously with the hybridisation step, the preparation may be treated to separate the double-
35 stranded target. This separation can be achieved by heating the preparation in the presence of a denaturing

agent such as formamide. The detection of a specific nucleic acid sequence in a chromosome may also be carried out using chromosomes in interphase, e.g. in tissue sections or in a suspension of cells. In such cases, the
5 specimens may be treated as described above for tissue sections or suspension of cells.

For all sample preparations, the nucleic acids are fixed in a morphological structure allowing hybridisation to be
10 carried out *in situ*. Thus, the nucleic acids are not extracted from the cellular material and the hybridisation is not carried out in solution.

Peptide nucleic acids to be used in the present method

15 The term "naturally occurring nucleobases" includes the four main DNA bases (i.e. thymine (T), cytosine (C), adenine (A) and guanine (G)) as well as other naturally occurring nucleobases (e.g. uracil (U) and hypoxanthine).

20 The term "non-naturally occurring nucleobases" comprises, e.g., naturally occurring nucleobases wherein a label is coupled to the base optionally through a suitable linker, and modified naturally occurring nucleobases such as,
25 e.g., modified uracil and hypoxanthine. Other examples of non-naturally occurring nucleobases are 2,6-diamino purine, propynylcytosine (C propynyl), isocytosine (iso-C), isopseudocytosine (iso^{pseudo}C), 5-methyl-isocytosine (iso¹⁴C), and thiouracil (see e.g. Tetrahedron Letters
30 Vol 36, No 21, 3601-3604 (1995) (ref. 6)).

Examples of useful intercalators are e.g. acridin, anthraquinone, psoralen and pyrene.

35 Examples of useful nucleobase-binding groups are e.g. 3-nitro pyrrole and 5-nitro indole.

In the present context, "C₁₋₄ alkyl" is intended to mean branched or non-branched alkyl groups containing 1 to 4 carbon atoms. Non-limiting examples are CH₃, CH₂CH₃ and
5 CH(CH₃)₂.

Within the present context, the expression "naturally occurring amino acid" is intended to comprise D- and L-forms of amino acids commonly found in nature, e.g. D-
10 and L-forms of Ala (alanine), Arg (arginine), Asn (asparagine), Asp (aspartic acid), Cys (cysteine), Gln (glutamine), Glu (glutamic acid), His (histidine), Ile (isoleucine), Leu (leucine), Lys (lysine), Met (methionine), Phe (phenylalanine), Pro (proline), Ser (serine),
15 Thr (threonine), Trp (tryptophan), Tyr (tyrosine) and Val (valine).

In the present context, the expression "non-naturally occurring amino acid" is intended to comprise D- and L-forms of amino acids other than those commonly found in
20 nature as well as modified naturally occurring amino acids. Examples of useful non-naturally occurring amino acids are D- and L-forms of Cha (cyclohexylalanine), Cit (citrulline), Hci (homocitrulline), HomoCys (homocystein), Hse (homoserine), Nle (norleucine), Nva (norvaline), Orn (ornithine), Sar (sarcosine) and Thi (thienylalanine).
25

The peptide nucleic acid probes to be used in the present
30 method should comprise a sufficient number of ligands capable of interacting with the nucleotide sequence to be determined to form hybrids sufficiently stable under the stringency used in the hybridisation and in the post-hybridisation wash. The strategy for selecting the
35 ligands of the peptide nucleic acid probe to be used in

accordance with the present method may be based on available target nucleotide sequence information.

In the above-indicated peptide nucleic acid probes, the backbone of the moieties may preferably consist of six atoms. This has been shown to provide the presently strongest observed affinity for nucleic acids. It may in some cases be advantageous to change the strength of the binding between the peptide nucleic acid probe and the nucleic acid sequence. Such change of the affinity may be accomplished by separating the ligands by fewer or by more atoms than six atoms. It is contemplated that preferred peptide nucleic acid probes to be used in the present method comprise less than 25% by weight (calculated excluding X and Q groups as well as any linkers and/or labels) of moieties having more or less than six atoms in the backbone.

The strength of the binding between the peptide nucleic acid probe and the nucleic acid sequence is influenced by the ligand Q. Hoogsteen and/or Watson-Crick base pairing assist in the formation of hybrids between a nucleic acid and a peptide nucleic acid probe wherein Q designates a nucleobase. It is contemplated that one or more of the ligands may be a group which contribute little or none to the binding of the nucleic acid such as hydrogen. It is contemplated that peptide nucleic acid probes to be used in the present method comprise less than 25% by weight moieties wherein Q designate H. One or more of the ligands Q may be groups that stabilise nucleobase stacking such as intercalators.

In the above-indicated peptide nucleic acid probes, one or more of the Q-groups may designate a label. Examples of suitable labels are given below. Moieties wherein Q denotes a label may preferably be located in one or both

of the terminating moieties of the peptide nucleic acid. Moieties wherein Q denotes a label may also be located internally.

- 5 The peptide nucleic acid comprises polymerised moieties as defined above. From the formula, it is to be understood that the peptide nucleic acid probe may comprise polymerised moieties the structure of which may be mutually different or identical.

10

The backbone of the peptide nucleic acid probe may suitably form a polyamide comprising polymerised moieties, wherein all X groups are O, or form a polythioamide comprising polymerised moieties, wherein
15 all X groups are S. The polyamide and polythioamide forming moieties may be linked to form probes comprising both polyamide and polythioamide forming moieties or to form probes comprising polyamide forming moieties or polythioamide forming moieties only. Peptide nucleic acid
20 probes having a polyamide backbone (all X groups designate O) are of particular interest.

The preferred length of the peptide nucleic acid probe will depend on the target material and whether labelled
25 peptide nucleic acid probes are used. It is contemplated that especially interesting peptide nucleic acid probes comprise from 8 to 40 polymerised moieties as defined above. Peptide nucleic acid probes comprising from 8 to 30, from 12 to 25, or from 12 to 20 polymerised moieties
30 may be of particular interest, and peptide nucleic acid probes comprising from 14 to 23, and from 14 to 20 moieties are of most interest.

As mentioned above, the polymerised moieties of the
35 peptide nucleic acid probe may be mutually different or identical. In some embodiments, the polymerised moieties

of formulas (IV)-(VII) constitute at least 75% by weight (calculated as defined above), preferably at least 80% by weight and most preferably at least 90% by weight of the probe.

5

The ends on the moieties terminating the peptide nucleic acid may be substituted by suitable substituents. The substituents may be chosen so as to form the free or acylated form of the terminating moiety. The substituents may further be chosen so as to form an ester, an amide or an amine depending on the chemical nature of the terminating moiety. A terminating end may further be substituted by one or more labels, which labels may be incorporated end to end, i.e. so as to form a non-branched labelled end, or may be incorporated so as to form a branched labelled end ("zipper"). A terminating end may further be substituted by one or more linker units. Such linker units may be attached directly to a terminating end, may be attached to a label or between labels on a terminating end, or be attached to a terminating end before a label is attached to a terminating end. It should be understood that two terminating ends may carry different or identical substituents, linker units and/or labels. It should further be understood that the term "a label" is intended to comprise one or more labels.

The expression "peptide label" is intended to mean a label comprising from 1 to 20 naturally occurring or non-naturally occurring amino acids, preferably from 1 to 10 naturally occurring or non-naturally occurring amino acids, more preferably from 1 to 8 naturally occurring or non-naturally occurring amino acids, most preferably from 1 to 4 naturally occurring or non-naturally occurring amino acids, linked together end to end in a non-branched or branched ("zipper") fashion. In a preferred

embodiment, such a non-branched or branched end comprises one or more, preferably from 1 to 8 labels, more preferably from 1 to 4, further labels other than a peptide label. Such further labels may suitably terminate
5 a non-branched end or a branched end. One or more linker units may suitably be attached to the terminating end before a peptide label and/or a further label is attached. Such linker units may also be attached between a peptide label and a further label.

10

The probe as such may also comprise one or more labels such as from 1 to 8 labels, preferably from 1 to 4 labels, and/or one or more linker units, which may be attached internally, i.e. to the backbone of the probe.
15 The linker units and labels may mutually be attached as described above.

In the present context, the term "label" refers to a substituent which is useful for detection of hybrids
20 formed between a peptide nucleic acid probe and a nucleic acid. In accordance with the present invention, suitable labels comprise fluorophores, biotin, dinitro benzoic acid, digoxigenin, radioisotope labels, peptide or enzyme labels, dyes, chemiluminescence labels, hapten, antigen
25 or antibody labels, and spin labels. Examples of particular interesting labels are biotin, fluorescent labels, such as fluorescein labels, e.g. 5-(and 6)-carboxyfluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid and
30 fluorescein isothiocyanate, peptide labels, dinitro benzoic acid, rhodamine, tetramethylrhodamine, cyanine dyes such as Cy2, Cy3 and Cy5, optionally substituted coumarin, R-phycoerythrin, allophycoerythrin, Texas Red and Princeton Red as well as conjugates of R-
35 phycoerythrin and, e.g. Cy5 or Texas Red.

Examples of preferred labels are biotin, fluorescent labels, peptide labels and dinitro benzoic acid. Peptide labels may preferably be composed of from 1 to 10, more preferably of from 1 to 8, most preferably of from 1 to 4, naturally occurring or non-naturally occurring amino acids. It may be particularly advantageous to incorporate one or more other labels as well as a peptide label such as from 1 to 8 or from 1 to 4 other labels. Two of such other labels may e.g. be incorporated at each terminating end.

Suitable peptide labels may preferably be composed of cysteine, glycine, lysine, ornithine, glutamic acid or aspartic acid.

15

A linker may be made up of linker units selected from units of formulas $\text{-NH(CH}_2\text{CH}_2\text{O)}_n\text{CH}_2\text{C(O)-}$, $\text{-NH(CHOH)}_n\text{C(O)-}$, $\text{-(O)C(CH}_2\text{OCH}_2)_n\text{C(O)-}$ and $\text{-NH(CH}_2)_n\text{C(O)-}$, wherein n is 0 or an integer from 1 to 8, preferably from 1 to 3. A linker unit may have a free amino group or a free acid group, i.e.

$\text{NH}_2(\text{CH}_2\text{CH}_2\text{O)}_n\text{CH}_2\text{C(O)-}$, $\text{NH}_2(\text{CHOH)}_n\text{C(O)-}$,
 $\text{HO(O)C(CH}_2\text{OCH}_2)_n\text{C(O)-}$, $\text{NH}_2(\text{CH}_2)_n\text{C(O)-}$, $\text{-NH(CH}_2\text{CH}_2\text{O)}_n\text{-CH}_2\text{C(O)OH}$,
 $\text{-NH(CHOH)}_n\text{C(O)OH}$, $\text{-(O)C(CH}_2\text{OCH}_2)_n\text{C(O)OH}$ and $\text{-NH(CH}_2)_n\text{C(O)OH}$. A linker may consist of up to 3 of such linker units. Examples of very interesting linker units are $\text{-NHCH}_2\text{C(O)-}$, $\text{-NHCH}_2\text{CH}_2\text{C(O)-}$, $\text{-NH(CH}_2\text{CH}_2\text{O)}_2\text{CH}_2\text{C(O)-}$, $\text{HO(O)CCH}_2\text{C(O)(NH(CH}_2\text{CH}_2\text{O)}_2\text{CH}_2\text{C(O))}_2\text{-}$.

In a further embodiment, the ligand Q as defined above may be labelled. Suitable labels are as defined above. Between such a label, a linker selected from C_{1-15} alkyl, C_{1-15} alkenyl and C_{1-15} alkynyl may be incorporated. In the present context, " C_{1-15} alkyl, C_{1-15} alkenyl and C_{1-15} alkynyl" are intended to mean branched or non-branched alkyl, alkenyl and alkynyl groups containing from 1 to 15 carbon atoms. It is preferred that such labelled ligands

Q are selected from thymine and uracil labelled in the 5 position.

In a preferred embodiment, probes used are peptide
5 nucleic acid probes comprising polymerised N-(2-amino-ethyl)glycine moieties of formula (VII) wherein the glycine nitrogen is connected to naturally occurring nucleobases by a methylene carbonyl linker. The peptide
10 nucleic acid probes to be used as detection probes may suitably comprise from 8 to 40, from 8 to 30 of such polymerised moieties, preferably from 12 to 25 or 12 to 20 moieties, most preferably from 14 to 23 or from 14 to 20 moieties.

15 Nucleobase sequence of the peptide nucleic acid probes

The nucleobase sequence of the peptide nucleic acid probes can be obtained by analysing the nucleobase sequence of the target sequence.

20

The nucleobase sequence of interesting genes can e.g. be obtained from the GenBank Database, or the information can be obtained by sequencing interesting genes.

25 The nucleobase sequence of each set of hybridisation probes is selected having regard to the following: Minimal degree of hairpin formation should be possible, minimal degree of self dimer formation should occur, and minimal degree of pair dimer formation should occur.
30 Prospected probe sequences are compared with sequences obtained from relevant data bases like the ones available via URL:<http://www.NCBI.NLM.NIH.gov>.

35 Preparation of peptide nucleic acid probes for hybridisation

The peptide nucleic acid probes used in the examples were synthesised according to the procedures described in "PNA Information Package" obtained from Millipore Corporation (Bedford, MA, USA).

5

If using the Fmoc strategy for elongation of the peptide nucleic acid probe with linkers or amino acids, it was possible to have side chain amino groups protected with acid sensitive protection groups such as the Boc, the Mmt or the Mtt group.

10

One way of labelling the peptide nucleic acid probe is to use a fluorescent label, such as 5-(and 6)-carboxy-fluorescein, 5- or 6-carboxyfluorescein, 6-(fluorescein)-5-(and 6)-carboxamido hexanoic acid or fluorescein isothiocyanate. The acid group is activated with HATU (O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and reacted with the N-terminal amino group or other side-chain or linker amino group. The same technique can be applied to other labelling groups containing an acid function. Alternatively, the succinimidyl ester of the above-mentioned labels may be used directly.

15

20

25

After synthesis, the peptide nucleic acid probes are cleaved from the resin using standard procedures as described by Millipore Corporation or PerSeptive Biosystems. The peptide nucleic acid probes can be purified and analysed using reversed-phase HPLC techniques at 50°C and were characterised by matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOFMS), plasma desorption mass spectrometry (PDMS) or electron spray mass spectrometry (ESMS).

30

35

Generally, peptide nucleic acid probes such as peptide nucleic acid probes comprising polymerised moieties of formula (IV)-(VII) may also be prepared as described in, e.g., Tetrahedron Letters Vol 35, No 29, 5173-5176 (1994) (ref. 7). Chemical properties of some peptide nucleic acid probes are described in, e.g., Nature, 365, 566-568 (1993) (ref. 8).

Hybridisation

10

The hybridisation can be performed using fixed, immobilised or suspended preparations which are prepared as described above. If double-stranded target such as chromosomal or other DNA sequences are to be detected, a treatment to separate the two strands may be necessary. This separation of the strands can be achieved by heating the sample in the presence of the hybridisation mixture to a temperature sufficiently high and for a time period sufficiently long to dissociate the strands. Typically, heating at a temperature of 70°C to 100°C for a period of 1 to 15 minutes is suitable.

The hybridisation buffer may optionally comprise a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between the nucleic acid to be determined and the peptide nucleic acid probe so as to increase the ratio between specific binding and non-specific binding. This agent will allow the hybridisation to take place at a lower temperature than without the agent. In traditional nucleic acid hybridisation, such agent is called a denaturing agent.

The effective amount of the hybrid destabilising agent will depend on the type of destabilising agent used and furthermore on the peptide nucleic acid probe or combination of peptide nucleic acid probes used. It has

been found that using peptide nucleic acid probes of formula (VII), particular good results have been obtained by including a hybrid destabilising agent. Examples of hybrid destabilising agents are formamide, urea, guanidine, ethylene glycol and glycerol, and most of these agents may preferably be used in a concentration above 10% and less than 80%, such as 70%. Formamide and guanidine may more preferably be present in an amount of from 20% to 80%, more preferably of from 20% to 60%, most preferably of from 30% to 70% or from 30% to 50%. Urea is preferably used in a concentration of from 2 to 5 M. Ethylene glycol may more preferably be present in an amount of from 30% to 65%, most preferably of from 50% to 65%.

It is often advantageous to include macromolecules or polymers such as dextran sulphate, polyvinylpyrrolidone and/or ficoll. In the presence of such macromolecules or polymers, the effective concentration of the peptide nucleic acid probe at the target is assumed to be increased. Dextran sulphate may be added in a concentration of up to 15%. Concentrations of dextran sulphate of from 2.5% to 12.5% are often advantageous.

In some cases, it may be advantageous to add a detergent such as sodium dodecyl sulphate, Tween 20® or Triton X-100®.

During hybridisation, other important parameters are hybridisation temperature, concentration of the peptide nucleic acid probe and hybridisation time. The person skilled in the art will readily recognise that optimal conditions for various starting materials will have to be determined for each of the above-mentioned parameters.

The hybridisation between peptide nucleic acid probes and a target nucleic acid sequence appears to be less sensitive than DNA probes to variations in pH and in the concentration of NaCl.

5

Post-hybridisation washing

Following hybridisation, the preparation is washed to remove any unbound and any non-specifically bound peptide
10 nucleic acid probes. The conditions described below are merely by way of example and may depend on the type of preparation to be analysed. During the post-hybridisation step, appropriate stringency conditions should be used in order to remove any non-specifically bound peptide
15 nucleic acid probe. Stringency refers to the degree to which reaction conditions favour the dissociation of the formed hybrids and may be enhanced, for instance by increasing the washing temperature and washing time. For conventional hybridisation with nucleic acid probes, the
20 salt concentration is often used as an additional factor for regulating the stringency.

Examples of useful buffer systems are Tris-Buffered-Saline (TBS), standard citrate buffer (SSC) or phosphate
25 buffers. A convenient TBS buffer is 0.05 M Tris/HCl, 0.15 M NaCl, pH 7.6. The SSC buffer comprises 0.15 M sodium chloride and 0.015 M trisodium citrate, pH 7.0. Typically, washing buffers include a detergent such as sodium dodecylsulphate, Tween 20[®], or Triton X-100[®].

30

Typically, washing times from 5 to 30 minutes may be suitable. Washing periods of two times 10 minutes or 3 times 5 minutes in a suitable buffer may also give good results.

35

In some cases, particularly when using peptide nucleic acid probes carrying at least one fluorescein label, it has been shown to be advantageous to increase the pH of the washing buffer. In such cases, it is preferred that
5 the washing solution in step (c) has a pH value of from 8 to 10.5, preferably from 9 to 10.

Detection of hybrids formed

10 In cases where the preparation is deposited onto slides, the hybridisation results may be visualised using well known immunohistochemical staining methods to detect the labelling on the peptide nucleic acid probe. When
15 fluorescent labelled peptide nucleic acid probes are used, the hybrids may be detected using an antibody against the fluorescent label which antibody may be conjugated with an enzyme. The fluorescent label may alternatively be detected directly using a fluorescence
20 microscope, or the results may be automatically analysed on a fluorescent-based image analysis system.

When biotin labelled peptide nucleic acid probes are used, the hybrids may be detected using enzyme labelled streptavidin or antibodies against the biotin label which
25 antibody may be conjugated with an enzyme. If necessary, an enhancement of the signal can be generated using commercially available amplification systems such as the catalysed signal amplification system for biotinylated probes (DAKO K 1500). Digoxigenin labelled probes may be
30 detected using antibodies against digoxigenin, and dinitro benzoic acid with antibodies against dinitro benzoic acid.

In the case of a suspended sample such as a cell
35 suspension, quantitative results may be obtained using peptide nucleic acid probes comprising a fluorescent

label and a flow cytometer to record the intensity of fluorescence per cell. Signal amplification may be relevant for flow as well.

5 Peptide nucleic acid probes used in some aspects of the present method may form nucleic acid/peptide nucleic acid probe hybrids which can be recognised by an antibody described in WO 95/17430 (ref. 9). Hybrids formed between
10 the peptide nucleic acid probe, nucleic acid and the antibody can be detected in a direct immunohistochemical staining method using, for instance an enzyme conjugated form of the antibody, followed by detection of the enzyme activity or by the application of well known indirect immunohistochemical staining techniques.

15

ILLUSTRATIVE EMBODIMENTS

ILLUSTRATIVE EMBODIMENT 1

20 The method of the invention can suitably be used for detecting chromosome aberrations in ALL and NHL. The sets of hybridisation probes are chosen so as to flank each site of the potential breakpoint. Interesting regions are indicated below in Tables 1 and 2. Apart from the two
25 sets flanking the potential breakpoint, at least one set of probes targeting fusion partner genes may be included

TABLE 1. Chromosome aberrations in ALL.

Type of ALL	Chromosome aberration	
	Type	Involved genes
Precursor-B-ALL	t(1;19)(q23;p13)	E2A-PBX1
	t(4;11)(q21;q23)	MLL-AF4
	t(9;22)(q34;q11)	BCR-ABL
	t(12;21)(p13;q22)	TEL-AML1
T-ALL	TAL1 deletion	SIL-TAL1
	t(1;14)(q34;q11)	TAL1-TCRD
	t(10;14)(q24;q11)	HOX11-TCRD
	t(11;14)(p13;q11)	RHOM2-TCRD

TABLE 2. Chromosome aberrations in NHL.

Type of NHL	Chromosome aberration	
	Type	Involved genes
Follicular NHL	t(14;18) 3q37	IGH-BCL2 BCL6
Diffuse large cell centroblastic NHL	t(14;18) 3q27	IGH-BCL2 BCL6
Large cell anaplastic NHL	t(2;5)	NPM-ALK
Mantle cell NHL	t(11;15)	BCL1-IGH
Immunoblastic NHL	t(14;18)	IGH-BCL2
Burkitt's NHL	t(8;14) t(2;8) t(8;22)	MYC-IGH IGK-MYC IGL-MYC
Lymphocytic NHL		
Immunocytoma		
MALT-NHL		
Peripheral T-NHL		
Lymphoblastic T- NHL	TAL1 deletion t(1;14) t(10;14) t(11;14)	SIL-TAL1 TAL1-TCRD HOX11-TCRD RHOM1-TCRD

ILLUSTRATIVE EMBODIMENT 2

5

Determination of translocation involving the major
breakpoint (mbr) in the MLL gene (ALL-1) and HRX

10

The nucleobase sequences of the peptide nucleic acid probes are selected from EMBL + GenBank, Accession numbers HSALL1X28 (exon 28) and HSALL1X4 (exon 4). The nucleobases are chosen so as to form two sets of probes, a set flanking each site of the mbr region, e.g. from

exon 4 and exon 28. Each set of hybridisation probes consists suitably of from 1 to 500, from 1 to 250, from 1 to 100, or from 1 to 35 peptide nucleic acid probes.

- 5 The nucleobase sequence of the probes indicated below are selected from the lower strand (i.e. they hybridise to the upper strand). The position of the nucleobases relative to the whole target sequence are indicated as "position". The first number corresponds to the position
10 of the 5' end of the probe in the sequence, and the last number corresponds to the position of the 3' end of the probe.

Two suitable sets of probes are e.g.

15

	<u>ALL-1 EXON 4 (one set)</u>	<u>Position</u>	
	GGGAAAACACAGATGGAT	120-103	Seq ID No 1
	GATTTGGTCTCTGATTTATTTAG	145-123	Seq ID No 2
	GTTATTTTGGATTTTACTCCAG	235-214	Seq ID No 3
20	GGTAACTCTGAAATGTCCTT	243-262	Seq ID No 4
	TTTTTCAGGCTATCTTCTT	292-274	Seq ID No 5
	CTTAACTTAGACTTGAGAGGA	386-365	Seq ID No 6
	CCCCTCCTTCCTATTT	416-400	Seq ID No 7
	CCTTCCTCTCCGTCGTA	443-427	Seq ID No 8
25	GGGTCTTTATCCTTTCTGT	471-453	Seq ID No 9
	ACTTTCCTGGGGCTTTTC	517-501	Seq ID No 10
	TGTCCTTCCTTGCTTTC	539-521	Seq ID No 11
	AACTGTCTTATCTTCTTTTGTA	569-548	Seq ID No 12
	CTTCGAGGGCTTTGTCT	589-573	Seq ID No 13
30	GCATCTGTCCTTTTGGAA	634-617	Seq ID No 14
	CTTCTTTTTCAATTTCTTTT	699-679	Seq ID No 15
	GGGGCACTGAATCTACTAT	889-871	Seq ID No 16
	AGCTGCACTTGATTTTTC	923-906	Seq ID No 17
	CGAATATTTTGGACCTGTG	748-730	Seq ID No 18
35	GGTCATAATCCTCATCCTC	834-816	Seq ID No 19
	AGGAGATAGCACTGACAACA	783-764	Seq ID No 20

TTTGAGAGGAGTGCTGAGA	942-924	Seq ID No 21
TATCAACACTGGGGCTACTA	965-984	Seq ID No 22

	<u>ALL-1 EXON 28 (another set)</u>	<u>Position</u>
5	TACTGTGACGCCTGGAG	123-107
	AGTGGTTGGGGTAGGAC	50-34
	GCTGGGGTGATAAGGAA	144-128
	CGGTGGTGGAGACTGA	225-210
	TTGAAGAGGTGGAAGTGT	327-309
10	CACTGGACTGCTTCATTTC	411-393
	ACTCAGCACTTTGGTCTTC	470-452
	AAGGAGCCGATTTTACTA	569-586
	GAGGTGTGGGAAGGAGA	644-628
	GTTTGCTGATTGGGTAGAA	704-686
15	ATGTTTCGTTGATAATGGAT	785-767
	CTTCCTCACCAGTTGTCAC	906-888
	CAAAACCTCATCCATAAAC	941-923
	GACTCCTGGCATAGAAAG	1025-1008
	TTCCGTGGTGCCTGTA	1084-1069
20	CCATTTTTTAGAGGTGTCAG	1122-1104
	CATTTTTTGGATTGACTCTC	1146-1128
	GCAGGACTACTTTCTTTCA	1168-1150
	CTGTGGGAGATGTTGACT	1203-1186
	TATTATTGGGGCTTGGTT	1266-1249

25

The probes can of course be selected from the upper strand (i.e. so as to be capable to hybridise to the lower strand).

30 The probes are preferably labelled. E.g. the set of probes for exon 4 may be labelled with one or more fluorescein labels, and the set of probes for exon 28 with one or more Cy3.

35 Step (a): Preparation of sample and pre-treatment

Metaphase chromosome spreads can be prepared from peripheral blood, bone marrow, amnion, chorion villus samples or tumour cells. If prepared from peripheral blood, a sample of whole blood collected in a glass tube
5 containing heparin or another anticoagulant is mixed with suitable growth medium. As an Example, 0,5 ml whole blood may be added to 10 ml RPMI 1640 medium supplemented with 20% (v/v) foetal calf serum, 2 mM glutamine, 100 U penicillin/streptomycin, 1% phytohemagglutinin and 5 U/ml
10 heparin. The sample is incubated in a CO₂ incubator, e.g. at 37°C for 72 h. The chromosomes are arrested in the metaphase of the mitosis, e.g. by adding colcemid (0.1 µg/ml) to the culture approximately 1½ hours before harvesting the cells.

15 The cells are collected by centrifugation and re-suspended in a hypotonic buffer, e.g. 60 mM KCl at room temperature for about 30 minutes. The sample is treated with a suitable fixative such as methanol:acetic acid
20 3:1. The treatment with fixative may be repeated several times. After treatment the suspensions are left at freezing temperatures such as at -20°C from 20 minutes to 3 days. Chromosome spreads are prepared by spotting a suitable amount of the suspension onto a clean slide and
25 air-drying the slide.

Prior to hybridisation it may be advantageous to submit the specimens on the slides to a pre-treatment including RNase treatment and/or additional fixation and/or
30 protease digestion. The following procedure may e.g. be applied:

- 1) Immerse slides in TBS 2 min.
- 2) Immerse slides in 3,7% formaldehyde in TBS for 2 min.
- 35 3) Wash slides 3x5 min. in TBS.

- 4) Immerse slides in proper dilution of Proteinase K for 10 min at room temperature.
- 5) Incubate slides in RNase 10-100µg/ml in 2xSSC for 20-60 min. at 37°C.
- 5 6) Wash 3x5 min. in TBS.
- 7) Dehydrate specimens in cold ethanol series (70%, 85% and 96%) and air dry them.

Step (b): Hybridisation

10

Prior to hybridisation, the chromosomal DNA is denatured. Such denaturing may be carried out by placing the slide in a solution of 2xSSC and 70% formamide at about 80°C for a short period of time such as 3-5 minutes.

15

Denaturation may be performed by adding the probes in proper dilution in the hybridisation buffer onto the slide containing the sample and covering it with a coverslip. The slides are then placed in a preheated incubator adjusted to 80°C for 3 min. Hybridisation may be performed by placing the slide in the dark at room temperature or higher temperatures (50-60°C may be suitable) for 5-60 min.. A suitable hybridisation buffer is 10 mM Tris pH 7.2 with 70% formamide containing conventional blocking reagent.

25

Step (c): Removal of any unbound and any non-specifically bound peptide nucleic acid probe

30 The slide may be rinsed in TBS, pH 7.4 or 0.1xSSC to remove the coverslips followed by wash for about 5-45 min. in an appropriate washing buffer e.g. 0.1 x SSC (15 mM NaCl, 1.5 mM sodium citrate pH 7.0) with 0.1% Triton X-100® or PBS with 0.1% Tween-20®.

35

Step (d): Detection of hybrids formed

If the bound probes are fluorescence labelled visible signals may be found by inspection of the slides in the fluorescence microscope at this step. Additional
5 amplification of the signal may be provided using some of the many different immunohistochemical/immunocytochemical amplification systems well known in the art. Non-fluorescent labelled probes may also be detected using
10 some of the many different immunohistochemical/-immunocytochemical amplification systems well known in the art.

Following appropriate amplification/detection, slides are mounted for inspection in the fluorescence microscope. A
15 mounting media may include counter stain such as DAPI. A successful hybridisation should preferably result in easily visible signals/spots on the chromosomes and within the interphase nuclei. There should preferably be no interfering non-specific hybridisation on the
20 chromosomes. The microscope should be correctly equipped for the labels used.

EXAMPLES

25 EXAMPLE 1

Sequence of peptide nucleic acid probes

One set of probes targeting HER-2 was selected (Accession
30 number emb|X03363|HSEB2R Human c-erb-B-2 mRNA). The probes were selected and numbered as described in Illustrative embodiment 2. The nucleobase sequences of the peptide nucleic acid probes were as follows:

35	<u>Position</u>	
	222-205	Seq ID No 43
	CAAGAGGGCGAGGAGGAG	

	GGTAGAGGTGGCGGAGCAT	325-307	Seq ID No 44
	TGGGCAGGTAGGTGAGTTC	373-355	Seq ID No 45
	GTGGGCAGGTAGGTGAGTT	374-356	Seq ID No 46
	GGCCAGGGCATAGTTGTC	519-502	Seq ID No 47
5	GTATTGTTTCAGCGGGTCTC	551-533	Seq ID No 48
	GCAGAGCTGGGGGTTC	660-644	Seq ID No 49
	GAGCCAGCTGGTTGTTCT	715-698	Seq ID No 50
	GCGGTTGGTGTCTATCAGT	738-720	Seq ID No 51
	AGGCCAGGCAGTCAGAGTG	937-919	Seq ID No 52
10	CCACGTCCGTAGAAAGGTA	1099-1081	Seq ID No 53
	GCAGGGGGCAGACGAG	1126-1111	Seq ID No 54
	GGGCTTGCTGCACTTCTCA	1185-1167	Seq ID No 55
	GCAGCCAGCAAACTCC	1275-1260	Seq ID No 56
	GAGCGGGGCAGTGTGG	1350-1334	Seq ID No 57
15	GAGTAGGCGCCATTGTGC	1499-1482	Seq ID No 58
	CTTGCAGGGTCAGCGAGTA	1513-1495	Seq ID No 59
	GGTGTATGGTGGATGAGG	1590-1572	Seq ID No 60
	AGCTTGGTGCGGGTTCC	1650-1634	Seq ID No 61
	CCGGTTGGCAGTGTGGAG	1671-1654	Seq ID No 62
20	CCTGGCATTACATACTCC	1848-1830	Seq ID No 63
	AGGTCAGTGAGCCATTCTG	1900-1882	Seq ID No 64
	GGAGAGGTCAGGTTTCACA	2001-1983	Seq ID No 65
	GAGTGGGTGCAGTTGATGG	2072-2054	Seq ID No 66
	CACCGCAGAGATGATGGAC	2148-2130	Seq ID No 67
25	TGAGGATCCCAAAGACCAC	2197-2179	Seq ID No 68
	ATGCCCTTGCTAGACTGTGC	2387-2369	Seq ID No 69
	AGCTGCACCGTGGATGTCA	2561-2543	Seq ID No 70
	GAGCCAGCCCGAAGTCT	2776-2760	Seq ID No 71
	CTTGCCGACATTCAGAGT	3077-3059	Seq ID No 72
30	TGGCCATGCGGGAGAAT	3115-3099	Seq ID No 73
	CAGTGAGCGGTAGAAGGTG	3198-3180	Seq ID No 74
	CGGTGCCTGTGGTGGAC	3317-3301	Seq ID No 75
	CATCTGGCTGGTTCACATA	3607-3589	Seq ID No 76
	TTGAAGGTGCTGGGTGGAG	3887-3869	Seq ID No 77
35	TCAAGCAGGAAGGAAGGTT	4099-4081	Seq ID No 78

All probes are labelled with one or two fluorescein labels. As linkers, one or two $\text{NH}_2\text{CH}_2\text{CH}_2(\text{COCH}_2\text{OCH}_2\text{CON}-(\text{CH}_2\text{CH}_2\text{OCH}_3)_2)\text{CH}_2\text{COOH}$ -linkers and/or one or two $\text{NH}_2-(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2\text{C}(\text{O})$ -linkers were used.

5

Another set of probes targeting the centromer of chromosome 17 was selected. These probes are included because the HER-2 gene in normal cells are located to chromosome 17, and aneuploidy is frequently observed in cancer cells. The nucleobase sequences of the peptide nucleic acid probes were as follows:

	AACGAATTATGGTCACAT	Seq ID No 78
	GGTGACGACTGAGTTTAA	Seq ID No 79
15	TTTGGACCACTCTGTGGC	Seq ID No 80
	AACGGGATAACTGCACCT	Seq ID No 81
	TTTGTGGTTTGTGGTGGA	Seq ID No 82
	AGGGAATAGCTTCATAGA	Seq ID No 83
	ATCACGAAGAAGGTTCTG	Seq ID No 84
20	CCGAAGATGTCTTTGGAA	Seq ID No 85
	AAAGAGGTCTACATGTCC	Seq ID No 86

All probes were labelled with one or two fluorescein labels. As linkers, one or two $\text{NH}_2\text{CH}_2\text{CH}_2(\text{COCH}_2\text{OCH}_2\text{CON}-(\text{CH}_2\text{CH}_2\text{OCH}_3)_2)\text{CH}_2\text{COOH}$ -linkers and/or one or two $\text{NH}_2-(\text{CH}_2\text{CH}_2\text{O})_2\text{CH}_2\text{C}(\text{O})$ -linkers were used.

25

The probes were synthesised according to standard PNA synthesis procedures.

30

Sample preparation, hybridisation, washing and detection are carried out as described above under Illustrative embodiment 2.

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CLAIMS

1. A method of determining the presence of chromosome
aberrations in a sample of eukaryotic origin using *in*
5 *situ* hybridisation, comprising the steps of

- a) producing a preparation of the sample, whereby the
sample will be subject to a fixation,
- 10 b) contacting said preparation with a hybridisation
solution comprising at least two sets of
hybridisation probes, at least one set comprising one
or more peptide nucleic acid probes capable of
hybridising to specific nucleic acid sequences
15 related to a potential aberration in a chromosome,
and at least one set comprising one or more peptide
nucleic acid probes capable of hybridising to
specific nucleic acid sequences related to another or
the same potential aberration in a chromosome, and
20 optionally a hybrid destabilising agent in an amount
effective to decrease the melting temperature of
hybrids formed between said nucleic acid sequences
and said peptide nucleic acid probes so as to
increase the ratio between specific and non-specific
25 binding,
- c) removing any unbound and any non-specifically bound
peptide nucleic acid probe, and
- 30 d) determining the presence of peptide nucleic acid
probe in the preparation.

2. A method according to claim 1, wherein each set of
hybridisation probes hybridise to specific nucleic acid
35 sequences related to the same potential chromosome
aberration.

3. A method according to claim 1 or 2, wherein the potential aberration is a potential breakpoint.

5 4. A method of determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in situ* hybridisation, comprising the steps of

10 a) producing a preparation of the sample, whereby the sample will be subject to a fixation,

15 b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

25 c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and

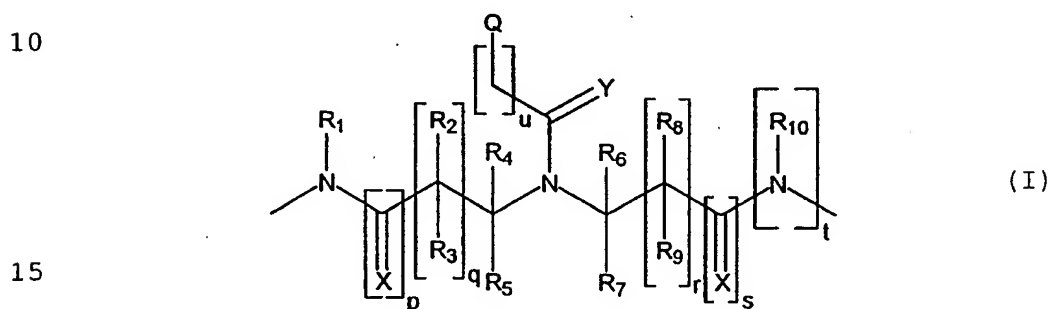
30 d) determining the presence of peptide nucleic acid probe in the preparation.

5. A method of determining the presence of chromosome aberrations in a sample of eukaryotic origin using *in situ* hybridisation, comprising the steps of

- 5 a) producing a preparation of the sample, whereby the sample will be subject to a fixation,
 - b) contacting said preparation with a hybridisation solution comprising at least two sets of
10 hybridisation probes, one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and one set comprising one or more peptide nucleic acid probes
15 capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic
20 acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,
 - c) removing any unbound and any non-specifically bound
25 peptide nucleic acid probe, and
 - d) determining the presence of peptide nucleic acid probe in the preparation.
- 30 6. A method according to claim 4 or 5, wherein each set of hybridisation probes hybridise to said specific nucleic acid sequences flanking a potential breakpoint in a chromosome within a genomic distance of no more than 100 kb, preferably no more than 50 kb from the potential
35 breakpoint.

7. A method according to any one of claims 1 to 6 wherein each peptide nucleic acid probe contains from 8 to 40 polymerised moieties.

5 8. A method according to any one of claims 1 to 7 wherein the peptide nucleic acid probe comprises polymerised moieties of formula (I)

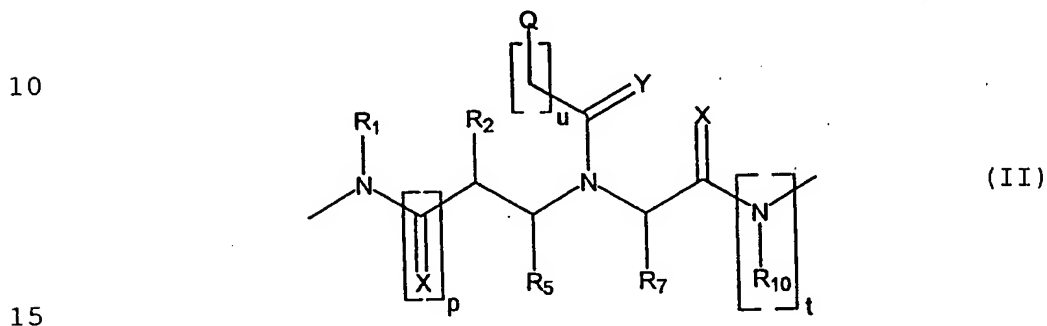


wherein

- 20 Y designates O or S,
 each X independently designates O or S,
 each Q independently designates a ligand that is a naturally occurring nucleobase, a non-naturally occurring nucleobase, an intercalator, a nucleobase-binding group,
 25 a label or H,
 u is an integer from 0, or 1 to 5,
 p and s independently designate 0 or 1,
 q and r independently designate 0 or 1,
 t designates 0 or 1,
- 30 R₁ and R₁₀ independently designate H or C₁₋₄ alkyl,
 R₂, R₃, R₄, R₅, R₆, R₇, R₈ and R₉ independently designate H, the side chain of a naturally occurring amino acid, or the side chain of a non-naturally occurring amino acid.
- 35 9. A method according to claim 8, wherein Y, X, Q, u, p, q, r, s, R₂, R₃, R₇ and R₈ are as defined in claim 8, t is

0, R_1 designates H or CH_3 , and each of R_3 , R_4 , R_6 and R_9 designates H.

10. A method according to claim 8, wherein the peptide
5 nucleic acid probe comprises polymerised moieties of
formula (II), which are moieties of the general formula
(I) wherein r is 0 and q and s are 1,



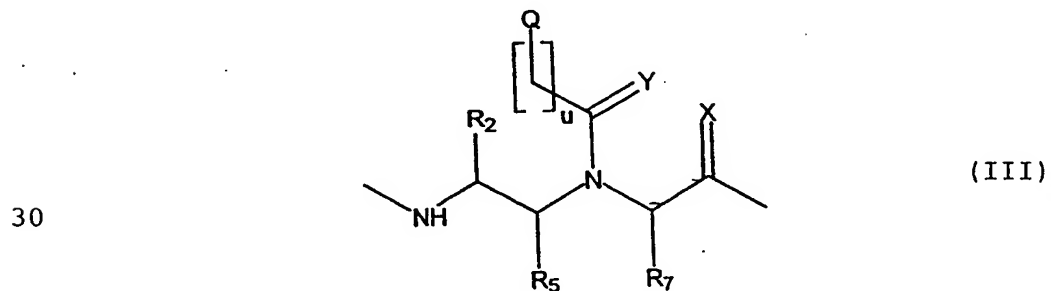
wherein Y , X , Q , p , t , u , R_2 , R_5 and R_7 are as defined in
claim 7, and

R_1 and R_{10} independently designate H or CH_3 .

20

11. A method according to claim 8, wherein the peptide
nucleic acid probe comprises polymerised moieties of
formula (III), which are moieties of the general formula
(I) wherein p , r and t are 0, and q and s are 1

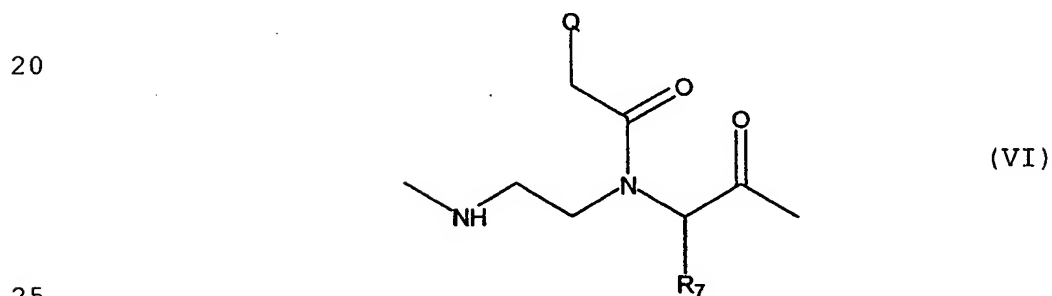
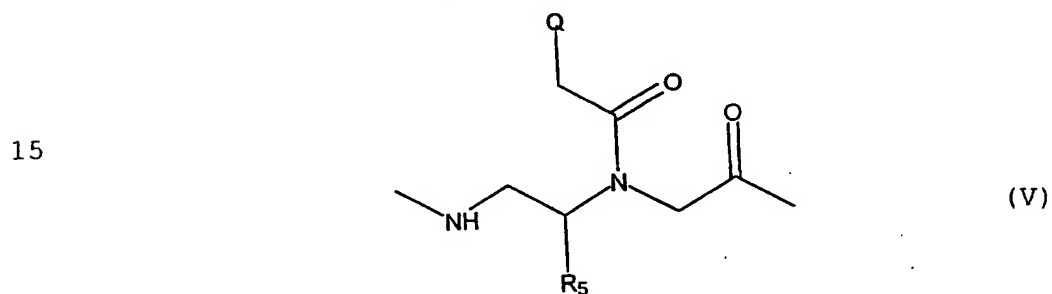
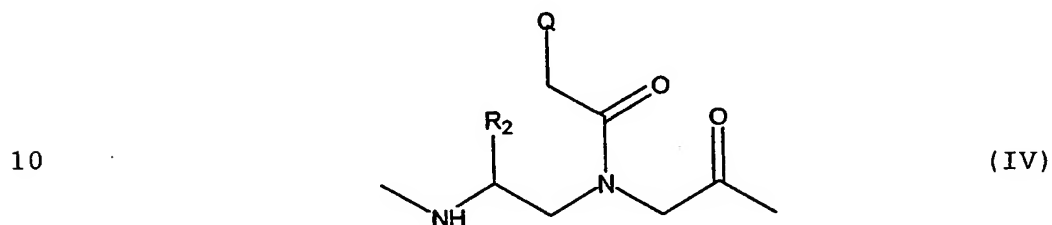
25



wherein Y , X , Q , u , R_2 , R_5 and R_7 are as defined in claim
8.

35

12. A method according to claim 8, wherein the peptide nucleic acid probe comprises polymerised moieties selected among those of formulas (IV)-(VI), which are moieties of the general formula (I) wherein p, r and t are 0, and u, s and q are 1,



wherein R_2 , R_5 and R_7 are as defined in claim 7, and each Q independently designates a naturally occurring nucleobase, a non-naturally occurring nucleobase, an intercalator or a nucleobase-binding group.

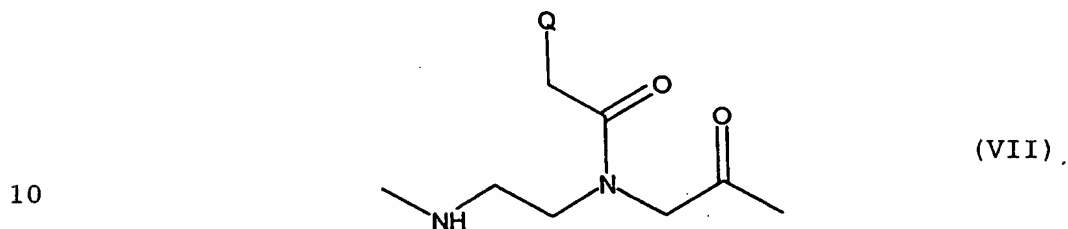
30

13. A method according to claim 12, wherein R_2 , R_5 and R_7 designate H or the side chain of Ala, Asp, Cys, Glu, His, HomoCys, Lys, Orn, Ser or Thr, and Q designates thymine, adenine, cytosine, guanine, uracil or hypoxanthine.

35

14. A method according to claim 8, wherein the peptide nucleic acid probe comprises polymerised moieties of formula (VII), which are moieties of formula (I) wherein p, r and t are 0, and u, s and q are 1:

5



wherein Q designates thymine, adenine, cytosine, guanine, uracil or hypoxanthine.

15

15. A method according to any one of claims 12 to 14, wherein polymerised moieties of formulas (IV)-(VII) constitute at least 75% by weight of the peptide nucleic acid probe.

20

16. A method according to any one of claims 1 to 15, wherein the hybrid destabilising agent is selected from the group consisting of formamide, urea, guanidine, ethylene glycol, and glycerol.

25

17. A method according to any one of claims 1 to 16, wherein the concentration of the hybrid destabilising agent is above 10%.

30

18. A method according to any one of claims 1 to 17 wherein each peptide nucleic acid probe is labelled directly or indirectly with at least one detectable label.

35

19. A method according to claim 18 wherein the label is selected from the group consisting of enzymes,

chromophores, fluorochromes, haptens, dyes and spin labels.

20. A method according to claim 19 wherein the label is
5 selected from the group consisting of fluorescein labels, biotin, digoxigenin, dinitro benzoic acid, peptide labels, rhodamine, R-phycoerythrine and cyanine dyes.

21. A method according to any one of claims 18 to 20
10 wherein the detectable label of one set of hybridisation probes is different from the detectable label of at least one other set of hybridisation probes.

22. A method according to any one of claims 1 to 21,
15 wherein two sets of hybridisation probes are used.

23. A method according to any one of claims 1 to 21,
wherein each set of hybridisation probes comprises from 1
to 500, from 1 to 250, from 1 to 100, or from 1 to 35
20 peptide nucleic acid probes.

24. A method according to any one of claims 1 to 23,
wherein the sample is a tissue section, a cell smear, a
cell suspension, or a chromosome spread.

25
25. A pair of sets of hybridisation probes, one set
hybridising to specific nucleic acid sequences related to
a potential aberration of a chromosome, and one set
hybridising to specific nucleic acid sequences related to
30 another or the same potential aberration of a chromosome,
each set comprising one or more peptide nucleic acid
probes of formula (I) to (VII).

26. A pair of sets of hybridisation probes according to
35 claim 25, each set capable of hybridising to nucleic acid

sequences related to different potential chromosome aberrations.

27. A pair of sets of hybridisation probes according to
5 claim 25 or 26, each set of hybridisation probes hybridising to said specific nucleic acid sequences related to the same potential aberration.

28. A pair of sets of hybridisation probes according to
10 claim 27, each set of probes hybridising to nucleic acid sequences related to a potential breakpoint.

29. A pair of sets of hybridisation probes, one set hybridising to specific nucleic acid sequences flanking
15 one side of a potential breakpoint in a chromosome and another set hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and each set comprising one or more peptide nucleic acid probes of formula (I) to (VII).

20 30. A pair of sets of hybridisation probes according to claim 29, each set hybridising to specific nucleic acid sequences flanking either side of a potential breakpoint in a chromosome within a genomic distance of no more than
25 100 kb, preferably no more than 50 kb from the potential breakpoint.

31. A pair of sets of hybridisation probes according to claim 25 to 30 characterised in that each set comprises
30 from 1 to 500, from 1 to 250, from 1 to 100, from 1 to 35 peptide nucleic acid probes.

32. An *in situ* diagnostic method for diagnosing chromosome aberrations in a sample of eukaryotic origin
35 comprising the steps of

- a) producing a preparation of the sample, whereby the sample will be subject to a fixation,
- b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to a potential aberration in a chromosome, and at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences related to another potential aberration in a chromosome, and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,
- c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and
- d) determining the presence of peptide nucleic acid probe in the preparation.

25

33. An *in situ* diagnostic method for diagnosing chromosome aberrations in a sample of eukaryotic origin comprising the steps of

- a) producing a preparation of the sample, whereby the sample will be subject to a fixation,
- b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, at least one set comprising one or more peptide nucleic acid probes capable of

35

hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and at least one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,

c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and

d) determining the presence of peptide nucleic acid probe in the preparation.

34. An *in situ* diagnostic method for diagnosing chromosome aberrations in a sample of eukaryotic origin comprising the steps of

a) producing a preparation of the sample, whereby the sample will be subject to a fixation,

b) contacting said preparation with a hybridisation solution comprising at least two sets of hybridisation probes, one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking one side of a potential breakpoint in a chromosome, and one set comprising one or more peptide nucleic acid probes capable of hybridising to specific nucleic acid sequences flanking the other side of the potential breakpoint and optionally a hybrid destabilising

- agent in an amount effective to decrease the melting temperature of hybrids formed between said nucleic acid sequences and said peptide nucleic acid probes so as to increase the ratio between specific and non-specific binding,
- 5
- c) removing any unbound and any non-specifically bound peptide nucleic acid probe, and
- 10 d) determining the presence of peptide nucleic acid probe in the preparation.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00245

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/68, C07H 21/00 // C12N 15/00, C12N 14/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9718325 A1 (DAKO A/S), 22 May 1997 (22.05.97), claims and page 5, lines 32-39 --	1-34
X	EP 0727487 A1 (K.U. LEUVEN RESEARCH & DEVELOPMENT), 21 August 1996 (21.08.96), page 4, lines 28-30, claims --	1-34
X	EP 0825198 A1 (K.U. LEUVEN RESEARCH & DEVELOPMENT), 25 February 1998 (25.02.98), page 5, lines 22-27 --	1-34

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search

28 July 1999

Date of mailing of the international search report

13. 09. 1999

Name and mailing address of the International Searching Authority
European Patent Office P.O. Box 6010 Postfach 2
NL-2280 HV Rijswijk
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 99/00245

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/DK 99/00245

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